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1 **Lessons from non-canonical splicing**

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Abstract

Recent improvements in experimental and computational techniques used to study the transcriptome have enabled an unprecedented view of RNA processing, revealing many previously unknown non-canonical splicing events.

- 5 This includes cryptic events located far from the currently annotated exons, and unconventional splicing mechanisms that have important roles in regulating gene expression. These non-canonical splicing events are a major source of newly emerging transcripts during evolution, especially when they involve sequences derived from transposable elements. They are therefore under precise
- 10 regulation and quality control, which minimises their potential to disrupt gene expression. While non-canonical splicing can lead to aberrant transcripts that cause many diseases, we also explain how it can be exploited for new therapeutic strategies.

Introduction

The vast majority of human genes contain more than one exon, and therefore introns need to be spliced from the nascent transcript and exons joined to form an mRNA that can be translated into a protein. Alternative splicing allows these exons to be joined in different variations to form **alternative transcripts**, which greatly increases the diversity of proteins encoded by a limited number of genes¹. This alternative splicing can be either enhanced or repressed by trans-acting factors, which are directed to the precursor mRNA by cis-acting regulatory elements^{1,2}. Genes producing long non-coding RNAs (lncRNAs) also typically contain multiple exons, and often display evidence for alternative splicing using similar mechanisms to those used for protein-coding mRNAs³

The mechanisms of splicing regulation and its perturbation in disease have been reviewed elsewhere^{4,5}. Here, our emphasis is on non-canonical splicing: this includes the cryptic splice sites that are located far from the currently annotated exons, and unconventional mechanisms that deviate from the well-defined rules of splicing. New methods to sequence the transcriptome, together with dedicated analysis pipelines (Box 1), have revealed a broad prevalence of non-canonical splicing events that can generate **cryptic exons**⁶⁻¹⁰, microexons¹¹⁻¹³, recursive splicing^{14,15}, circular RNAs (circRNAs)¹⁶⁻²⁰, retained introns²¹⁻²⁵ and exonic introns (exitrons)^{25,26}, among others. In this Review, we discuss the known mechanisms, functions and evolutionary potential of these events. We describe how mutations can cause disease by disrupting transcriptome integrity via non-canonical splicing, and how the cellular quality control systems defend the transcriptome from such perturbations. Finally, we explain how the unconventional splicing mechanisms can be targeted or exploited for new therapeutic strategies.

Types of non-canonical splicing

The fidelity of splicing is achieved by combinatorial recognition of specific sequences within precursor mRNA at many steps during the splicing process^{4,27}.

The first, and possibly the most important aspect of combinatorial recognition is described by the exon-definition model, which was proposed to explain how exons are recognised as functional units in metazoan organisms that contain long introns^{27,28}. This process involves interactions between factors bound to the flanking splice sites (e.g. U1 and U2 snRNPs, U2AF complex) and SR proteins bound to the exonic enhancer sequences. We will first discuss the cryptic exons, microexons and recursive splice sites (RS sites), which often require unconventional exon definition mechanisms. Next, we will discuss non-canonical splicing mechanisms that result from lower or higher splicing efficiency than normal (retained introns, exitrons), changes in the usual order of splicing (circRNAs, chimeric RNAs) or changes in the consensus sequence (atypical splice sites).

Cryptic splice sites and exons. Introns of ENSEMBL-annotated genes constitute around 23% of the human genome, and within such a vast sequence space it is inevitable that many sequences similar to the consensus motifs of canonical splice sites will be present by chance. Such sequences are known as cryptic splice sites. To prevent uncontrolled splicing at cryptic sites, exon definition mechanism has evolved to maintain splicing fidelity, which explains why most individual cryptic splice sites do not efficiently initiate splicing^{27,28}. Nevertheless, over half a million non-annotated splicing events have been discovered through the analysis of mouse and human RNA-seq data^{10,14,29-31}. Even though many of these events may be splicing ‘mistakes’ that are tolerated by the cell and have no function, targeted genome editing experiments are beginning to uncover functions of specific cryptic splice sites³¹. Moreover, cryptic splice sites can be present in a manner that can define non-annotated, or ‘cryptic exons’³². These exons often introduce premature termination codons (PTCs) into the resulting transcripts, which can target them for nonsense-mediated decay (NMD) in the cytoplasm³³⁻³⁵ (**Figure 1A**). In some cases, abnormal splicing also leads to transcription-coupled surveillance mechanisms that can decrease the expression of resulting transcripts³⁶. These quality control pathways often decrease the expression of transcripts containing cryptic exons, which makes these exons more difficult to detect and annotate during transcriptome sequencing analysis⁶.

Cryptic exons often emerge from transposable elements (TEs). In primates, antisense *Alu* sequences are the best substrates for the exonisation process, as they require a low number of mutations to form potent splice sites³⁷. The evolution of the *Alu* family consisted of two phases. The original *Alu* monomers arose from a fusion of the 5' and 3' ends of the 7SL RNA gene, which encodes an RNA component of the signal recognition particle (SRP). A further fusion of these monomers led to the modern *Alu* elements that are composed of left and right arms joined by an A-rich linker and followed by an A-tail (**Figure 1B**)³⁸. Notably, more than 330,000 *Alu* elements (annotated by RepeatMasker) are present in introns of protein-coding genes in an antisense orientation, where they are transcribed in a reverse orientation, thus containing two U-tracts instead of the A-tail and linker. These U-tracts can function as binding sites for splicing factors, especially U2 small nuclear RNA auxiliary factor (U2AF2) and T-cell intracellular antigen (TIA) proteins, which can induce the formation of cryptic or alternative *Alu* exons^{8,37,39}. Mechanisms regulating splicing of *Alu* exons and other cryptic exons have been uncovered with studies that map the binding sites of RNA binding proteins (RBPs) with cross-linking and immunoprecipitation (CLIP), individual nucleotide CLIP (iCLIP) and related techniques⁴⁰. The *Alu*-derived exons were found to be tightly repressed by heterogeneous nuclear ribonucleoprotein C (hnRNP C), which can displace U2AF2 from the long U-tracts (**Figure 1B**)⁸, and other cryptic exons were found to be repressed by NOVA⁷, RBP fox-1 homologue (*C. elegans*) 2 (Rbfox2)⁶ and TAR DNA binding protein 43 (TDP-43)⁹. Together with deep sequencing of RNA from tissues, these studies revealed thousands of previously unknown cryptic exons, and some of these are becoming recognised as regulated alternative exons¹⁰ (**Figure 1A**). Interestingly, repressive sequences were found to be more common at cryptic exons compared to the established alternative exons that emerged from transposable elements, indicating that loss of repression may have a role in the formation of new exons⁴¹.

Microexons. Exons that are shorter than 30 nt have traditionally been referred to as microexons⁴²⁻⁴⁴. New computational methods for analysis of sequencing data

revealed hundreds of previously unidentified microexons, 60% of which are preferentially included in neuronal tissues^{11-13,44}. Interestingly, microexons tend to be flanked by intronic motifs that are required for their inclusion, which bind to RBPs such as Serine/Arginine Repetitive Matrix 4 (SRRM4; also known as nSR100)¹², RBP Fox (RBFOX) or Polypyrimidine Tract Binding Protein 1 (PTBP1)¹³. SRRM4 is an SR-related protein that acts in an unusual way. Unlike other SR proteins that bind to exonic enhancers, SRRM4 binds to enhancers that are embedded within the unusually long polypyrimidine tract present upstream of microexons, thereby compensating for the limited space available for enhancer sequences within the microexons (**Figure 1C**).

Recursive splice sites. RS sites, also referred to as ‘zero-length exons’, are defined by a sequence that combines the 3' and 5' splice site consensus motifs. This allows an intron to be spliced in multiple consecutive steps: the 3' splice site is used to splice the preceding part of the intron, which reconstitutes a full 5' splice site that is then used to splice the remaining part of the intron (**Figure 2**). First discovered in the long introns of three *Drosophila melanogaster* genes^{45,46}, analyses using total RNA-seq and iCLIP identified 197 RS sites in *D. melanogaster*¹⁵ and 11 in human^{14,15}. In addition to detection of splice-junction reads bearing the RS site motif, these studies also required the presence of co-transcriptional splicing patterns (**Figure 2A**), which can only be reliably evaluated in long introns with high read coverage^{14,47,48} (Box 1). Accordingly, these numbers are probably underestimates. For instance, 419 cryptic splicing events were found at putative RS junctions in human samples prior to considering the co-transcriptional splicing patterns¹⁴. Notably, intrasplicing is another mechanism that can affect alternative splicing by using non-annotated splice sites. However, here the first splicing reaction reconstitutes a new 3' splice site, which can then be used by an upstream exon to remove the remaining intron⁴⁹.

Even though RS sites do not normally lead to splicing of an exon, they employ the exon definition mechanism in vertebrates (**Figure 2B**). These RS sites are present at the start of cryptic exons, referred to as RS exons¹⁴. Both the RS sites and the downstream 5' splice site that is required for exon definition are highly

conserved. Definition of the RS exon is essential to initiate splicing at the 3' splice site. After splicing of the preceding intron, the RS site reconstitutes a strong 5' splice site, which leads to skipping of the RS exon via recursive splicing. Whereas the exon definition mechanism is required for recursive splicing in human and zebrafish, it remains unclear how RS sites are defined in the fruit fly. The first RS sites discovered in the fruit fly overlapped with the start of annotated exons, indicating that an exon definition mechanism might be involved^{45,46}. Alternatively, the 3' splice site of intronic RS sites is often strongly conserved across *Drosophila*, which is consistent with the sensitivity of fruit fly recursive splicing to depletion of U2AF2¹⁵. It is also possible that RS sites are preceded by additional enhancer elements similar to microexons, which are flanked by binding motifs of multiple regulators, including SRRM4¹², RBFOX or PTBP1¹³.

Retained introns. Even though the precision and efficiency of splicing is very high, it is not perfect. Both in plants and animals, decreased efficiency of splicing at some introns can lead to their retention within polyadenylated transcripts^{21,25,50-52}. In fact, comparison of RNA from human and mouse tissues detected retained introns in alternative transcripts of most genes²¹⁻²⁴. Intron retention can be a result of various trans- and cis-acting mechanisms (**Figure 3A**). Most often, it is caused by an inefficient recognition of canonical splice sites⁵³. Under conditions of limiting spliceosome availability, such as upon downregulation of spliceosomal components, deficient splice site recognition can affect hundreds of introns in this way²⁴. Moreover, inclusion of shorter introns in mammalian cells can be more dependent on intron definition, a mechanism that brings the splice sites at both ends of the intron into closer proximity²⁷. This mechanism can be regulated by RBPs that bind at both ends of the intron and interact with each other⁵⁴. These proposed mechanisms agree with the generally weaker 5' and 3' splice sites, and shorter length of the retained introns compared to other introns^{21,53,55}. Moreover, retained introns have higher GC content compared to average introns, which might make them more sensitive to RNA polymerase II stalling^{21,53}. Certain RBPs can also promote specific intron retention events^{22,56,57}. For example, PTBP1 can repress recognition of a canonical splice site in an intron of the *FosB* gene⁵⁶, while Poly(A) Binding Protein, Nuclear 1 (PABPN1) promotes

retention of the last intron within its own transcript by binding to an adenosine-rich region in the 3' UTR⁵⁷. Finally, depletion of exon junction complex (EJC) components also leads to retention of long introns in *D. melanogaster*^{58,59}, although apparently not in human cells⁶⁰.

5 *Exitrons*. Some alternatively spliced introns are also present within regions annotated as exons. These introns are rarely spliced, and therefore they are referred to as cryptic introns, or also as 'exitrons'^{25,26} (**Figure 3B**). A total of 923 exitrons have been discovered within regions that are normally annotated as exons²⁶. Similar to retained introns, exitrons are shorter than average introns
10 and have weak splice sites, which can explain why they are retained under normal conditions. Exitrons are formed from exons that are amongst the longest known in humans, and have higher GC content than typical exons. They are formed when cryptic splice sites within an exon go on to pair with the canonical splice sites that flank the same exon, thereby leading to definition of two smaller
15 exons²⁶. Unlike retained introns, exitrons don't normally contain PTCs. Instead, their removal can change protein structure or lead to frame-shifts that introduce PTCs, which can target the resulting transcripts to NMD (**Figure 3B**).

Circular RNAs (CircRNAs). CircRNAs are formed as a result of pre-mRNA splicing that doesn't follow its canonical 5' to 3' order^{20,61}. The mechanism responsible
20 for this is referred to as back splicing, or head-to-tail splicing, where a branch point upstream of an exon attacks a downstream splice donor⁶²⁻⁶⁴. In some cases this happens with a single exon, whereas in others the start of an upstream exon splices to the end of a downstream exon, producing multi-exonic circRNAs¹⁷ (**Figure 4A**). In these multi-exonic circRNAs, the intervening intron can be
25 spliced out. We refer to such single- or multi-exonic circular transcripts that lack introns as 'exonic circRNAs'. Alternatively, if the intron between the exons remains retained, the resulting circular transcript is referred to as 'exon-intron circRNA'²⁰ (**Figure 4A**). Finally, 'intronic circRNAs' can be produced from intron lariats that are resistant to de-branching due to presence of C-rich motifs near
30 the branch point²⁰. These diverse types of circRNAs have been discovered in all domains of life^{16-20,65,66}. While most are quite rare, some are highly abundant in a specific tissue due to their resistance to exonucleases (**Figure 4B**)⁶⁷. Many have

tissue-specific expression patterns^{16,17,20,66}, and in the central nervous system they tend to be enriched within neuronal dendrites⁶⁸.

The head-to-tail splicing can be promoted by the presence of intronic inverted repeat sequences, which hybridise and thereby bring the ends of the relevant exons in proximity⁶⁹⁻⁷² (**Figure 4A**). In primates, hybridisation can be directed by inverted *Alu* repeats in flanking introns⁷⁰. As inverted *Alu* repeats are known to be a target for RNA editing, it is thus possible that formation of circRNAs could be regulated by editing. Indeed, dsRNA hybridisation sites that are edited by adenosine deaminase acting on RNA (ADAR) are seen in introns that flank circRNAs in *C. elegans*⁷³. However, formation of a dsRNA structure is not always required for circRNA formation⁶⁹. RBPs such as Quaking (QKI) and muscleblind-like (MBNL) proteins are also able to regulate circRNA biogenesis via binding sites in the flanking introns^{20,71,74,75} (**Figure 4A**).

Chimeric RNAs. Modified algorithms for analysis of RNA-seq data can identify chimeric RNAs, which are produced when splicing joins the exons of different genes (Box 1). Cis-splicing was proposed to result from deficient transcriptional termination, which allows proximal genes to be transcribed as a single unit, thereby resulting in splicing of the penultimate exon of the upstream gene to the second exon of the downstream gene⁷⁶. Such chimeric transcripts that combine exons of adjacent genes have been detected in several human tissues⁷⁷⁻⁷⁹ (**Figure 4C**). In contrast, trans-splicing joins exons derived from distant genomic locations (**Figure 4D**). The resulting chimeric transcripts have been best documented in trypanosomes, *C.elegans* and insects⁸⁰⁻⁸⁵, and to a lesser extent also in humans^{86,87}.

Atypical splice sites. More than 99% of human introns are spliced by the major U2-dependent spliceosome. Most 5' splice sites start with GTRAG, and remaining ones have a stronger preference for AG at the end of the exon, while 3' splice sites end with CAG, TAG or more rarely, AAG (**Figure 5A**). The introns spliced by the minor U12-dependent spliceosome can be distinguished by the longer consensus sequence at the 5' splice site and at the branch point^{88,89} (**Figure 5B**). Meanwhile, 5' splice sites that start with a GC are the most common atypical U2-

type splice sites^{90,91} (**Figure 5C**). Each 3' splice site is normally preceded by a branch point that contains an adenine nucleotide. It is common that multiple branch points are present⁹², and this can affect the choice of alternative 3' splice sites. This was recently demonstrated genome-wide following mutation of SF3B1
5 splicing factor⁹³⁻⁹⁵.

Multiple mechanisms could explain recognition of these atypical splice sites, including shifted base-pairing of small nuclear RNAs (snRNAs)⁹⁶ and bulged nucleotides that retain base-pairing to snRNAs⁹⁷. Some sites were also found to be modified by A-to-I RNA editing, in which inosine is effectively read as a
10 guanosine^{91,98}. An example of this mechanism is the ADAR2-dependent editing of an AA-3' dinucleotide within its own pre-mRNA, which then functions as a strong AG-3' splice site to change splicing of its transcript as part of an auto-regulatory mechanism⁹⁸. Finally, the unconventional cytoplasmic splicing of XBP1 and other
15 mRNAs during unfolded protein response, which employs the RNase Inositol-requiring enzyme 1 (IRE1) and RNA ligase RtcB, can create new exon-exon junctions that don't contain the standard consensus sequences⁹⁹⁻¹⁰¹.

The functions of non-canonical splicing

Non-canonical splicing events contribute to a great diversity of cellular
20 mechanisms and biological functions. Perhaps the best understood of these functions is that of microexons, which are enriched in genes associated with synapse biology and axonogenesis¹². Microexons are highly conserved, and their length generally comes in multiples of three, thereby preserving the open reading frame (ORF)¹¹⁻¹³. Microexons are enriched within modular interaction
25 domains, where they tend to encode charged residues that are accessible at the surface, and often overlap lipid or peptide binding domains^{12,13,102} (**Figure 1B**). It has been shown that inclusion of microexons alters the interactomes of several proteins¹². Owing to their common brain-specific splicing patterns, microexons have a major role in increasing proteome diversity in the brain. Therefore it is
30 not surprising that widespread skipping of microexons upon loss of SRRM4 in mice leads to neurodevelopmental defects¹⁰³. Another type of newly discovered

events that lead to new protein variants are exons, as most of these preserve the reading frame, and are enriched within disordered regions of the encoded proteins^{26,104} (**Figure 3B**).

Other forms of non-canonical splicing most often have a role in regulating gene expression. One of the best studied examples is the neuronal-expressed circRNA CDR1as/ciRS-7, which contains at least 63 conserved miR-7 binding sites that sequester this miRNA and thereby increases translation of its mRNA targets^{16,17}. Moreover, circRNAs can contribute to mechanisms that regulate transcription or splicing²⁰. For example, by enhancing production of circRNAs in its own transcript, the MBNL1 RBP decreases the amount of translation-competent transcripts produced from its own gene⁷⁴. In fact, many splicing factors regulate splicing of cryptic exons, introns or circRNAs in their own transcripts or those of other RBPs, as part of auto- or cross-regulatory mechanisms^{6,7,20,21,24,33-35,55,105} (**Figure 1A**). Retained introns often lead to retention of the host mRNA in the nucleus, where it undergoes exosome-mediated degradation²². If exported to the cytoplasm, most retained introns introduce PTCs, and may thereby promote NMD of the resulting transcript or lead to production of truncated proteins^{22,23,106,107} (**Figure 3A**). Intron retention was found to coordinate expression of related genes in granulocyte differentiation²⁴, at certain stages of the cell cycle⁵⁵ and across tissues²¹. Interestingly, intron retention is more common in transcripts that are less required for the physiology of a particular tissue²¹.

The function of recursive splicing in regulating gene expression remains to be fully understood. In human, RS sites are found in the extremely long introns of genes that are expressed mainly in the brain, and function in neuronal axon guidance and cell adhesion^{14,15}. It is tempting to speculate that recursive splicing could be important for splicing integrity of these introns. However, steric blocking of recursive splicing failed to reduce the overall splicing of the long intron in two human genes¹⁴. An alternative regulatory role was proposed for human RS sites¹⁴. These RS sites are followed by RS exons, which are spliced out of dominant isoforms, but included in minor isoforms that arise from use of upstream cryptic exons or rare alternative promoters (**Figure 2B**). The reason

for inclusion of RS exons in minor isoforms is that the preceding cryptic exons end with suboptimal sequences, and therefore they do not reconstitute a sufficiently strong 5' splice site at the RS site. Interestingly, most RS exons contain PTCs, and therefore their inclusion prevents translation of full-length proteins and targets the resulting transcripts to NMD (**Figure 2B**). It remains to be seen how many RS sites are involved in the regulation of alternative splicing.

Evolutionary perspectives on non-canonical splicing

Even though exemplary functions of individual non-canonical splicing events have been discussed in this review, the same function cannot be ascribed to all events of the same type. For example, even though a few circRNAs can sequester a miRNA, most of them are not abundant enough to have such a function¹⁰⁸. It is likely that many transcripts produced by non-canonical splicing have no function, and their presence reflects the capacity of cellular quality control mechanisms to protect from potential damaging effects of these transcripts. Most newly-emerging exons contain PTCs, and their initial emergence is likely to produce truncated or misfolded proteins that are likely to be deleterious for the organism. It is therefore not surprising that quality control mechanisms minimise the deleterious effects of such events. These include RBPs or snRNP complexes that have secondary activities aside from their usual roles in spliceosome function or regulation of canonical exons. These RBPs or snRNPs can repress splicing of cryptic exons^{8,74,75,109,110}, edit the nascent RNA to represses splicing^{111,112}, prevent mRNA export, or decrease the stability of aberrant mRNAs^{8,60,107,113,114}.

Many non-canonical events are introduced by transposable elements (TEs), which make up as much as two-thirds of the human genome¹¹⁵. For example, over 1.5 million degenerated long interspersed elements (LINE) sequences are annotated in human genome (<http://repeatmasker.org>), and while many are transcribed as parts of other genes, fewer than 100 of them are capable of retrotransposition¹¹⁶. This indicates that evolution constantly puts the degenerated TEs to new uses, and when present in transcribed regions, they are

a rich source of new exons and other elements for post-transcriptional control^{37,117,118}. The newly-emerging *Alu* exons are controlled by an antagonistic interplay between two RBPs, hnRNP C and U2AF2, which compete for binding to U-tracts, thereby affecting the splicing outcome⁸ (**Figure 1B**). While mutations
5 creating a splice site can cause a major increase in the inclusion of an *Alu* exon, mutations that change a single uridine within the U-tract are likely to only slightly modify the inclusion of *Alu* exons. Thus, repression by hnRNP C might ensure that new *Alu* exons emerge gradually, rather than in discrete steps¹¹⁹. Notably, hnRNP C is a conserved protein in vertebrates, and therefore it has
10 preceded the insertion of *Alu* elements into primate genomes. It remains unknown how such conserved RBPs controlled the transition of diverse classes of TEs within vertebrate genomes from a state of repressed TE-derived cryptic exons into functionally regulated alternative exons.

One way to explain the evolutionary functions of emerging non-canonical
15 splicing events is the multilevel selection theory¹²⁰. According to this theory, even if only a small number of individual species- or clade-specific TEs were beneficial at the level of organisms, the prevalence of TEs could have adaptive value for the species or clade by promoting speciation or preventing extinction. Similarly, the prevalence of cryptic splicing might increase the probability for
20 emergence of a few species-specific splicing events that can reset the gene regulatory networks. Such evolutionary tinkering is particularly important for complex organisms, in which it is linked to the increased size of the non-coding genomic regions¹²¹. Notably, the genes with the longest introns tend to be most highly expressed in the brain¹⁴, and these long introns produce the highest
25 number of non-canonical splicing events^{122,123}. It remains to be seen if and how such events may have contributed to the evolution of regulatory networks in the vertebrate brains.

By decreasing the expression of new transcript variants produced by non-canonical splicing, the cellular quality control pathways not only protect our cells
30 from their potentially toxic effects, but also decrease the negative selection against these variants during evolution. Thus, the low expression level of transcripts produced by non-canonical splicing provides an opportunity for

evolution to test the newly emerging variants and to select against toxic protein isoforms before expressing them at higher levels. It remains to be seen if established transcripts that generate functional protein isoforms created by alternative splicing in our cells might have initially emerged as cryptic splicing events in an ancestral species, and were then gradually co-opted by evolution for new functions.

Non-canonical splicing and disease

Disease-associated variations. Approximately a third of disease-causing mutations are presently estimated to disrupt pre-mRNA splicing¹²⁴⁻¹²⁶. This effect can occur either via mutations in *cis*-elements within pre-mRNAs, or via mutations or misregulation of *trans*-regulatory factors that bind to pre-mRNAs⁵. This figure may be an underestimate, since it does not include the disease-linked synonymous variants within exons that can affect splicing¹²⁷. Moreover, even though standard computational models focus on positions close to canonical splice sites to identify variants that might affect RNA splicing, new models are being developed that can predict variants at other positions¹²⁸. These models were successful in the analysis of variants in spinal muscular atrophy (SMA), colorectal cancer and autism spectrum disorder (ASD).

Mutations that are located far from canonical splice sites can activate non-canonical splicing to cause disease^{12,86,129-139}. For example, the core spliceosomal component U1 snRNP can repress cryptic exons when it binds in a non-productive conformation (**Figure 6A**). Deletion of the repressive U1 snRNP binding sites was found to activate splicing of cryptic exons in both ataxia telangiectasia and Laron syndrome^{110,140}. Generally, most studied mutations that induce splicing of cryptic exons achieve this by inactivating repressive sequences or secondary structures¹³³⁻¹³⁵ or increasing the strength of a cryptic splice site (**Figure 6B**). Moreover, disruption of canonical splicing can activate distal cryptic polyadenylation sites^{136,137} (**Figure 6B**). For example, triplet repeats

within the first exon of the HTT transcript inhibit splicing of the following intron, thereby activating a cryptic polyadenylation site within the intron¹³⁸. The resulting transcript can be translated into short toxic peptides that contribute to the molecular pathogenesis of Huntington disease.

5 About a half of the cryptic exons that are linked to disease are derived from TEs, particularly *Alu* elements, which have diverged from their original sequence by accumulating mutations that create splice sites^{37,141}. For example, a cryptic *Alu*-derived exon can disrupt expression of the *DMD* gene, thereby causing the Duchenne muscular dystrophy (DMD) phenotype¹³⁰. A particularly rich source of
10 variation within the antisense *Alu* elements are the U-tracts, which can control the formation of an *Alu* exon by affecting the competition between hnRNP C and U2AF2^{8,129}. This mechanism has been seen in the *PTS* gene, in which a >50nt deletion containing the U-tract leads to splicing of a cryptic *Alu* exon (**Figure 3C, 6A**), which disrupts *PTS* gene expression and leads to the neurological condition
15 hyperphenylalaninaemia¹²⁹.

In addition to cis-acting mutations, a changed activity of a trans-acting factor can perturb non-canonical splicing in a manner that leads to disease^{12,142-146}. A link between the reduced expression of *SRRM4* mRNA and decreased splicing of microexons was also observed in individuals with ASD¹² (**Figure 1B**). Moreover,
20 TDP-43, a major component of aggregates in ~50% of cases of frontotemporal dementia and ~98% of amyotrophic lateral sclerosis cases, was found to repress splicing of a large number of cryptic exons with potential relevance for disease mechanisms⁹. The resulting disease-associated inclusion of a cryptic exon into the autophagy-associated gene, *ATG4B*, might lead to the defects in autophagy
25 that are commonly linked to these diseases¹⁴⁷. In addition, mutations in components of the minor spliceosome can lead to specific diseases by causing retention of U12-type introns, without affecting U2-type introns^{142-146,148}.

The disease associations of other recently described non-canonical splicing events remain to be examined. While potential roles of circRNAs in disease have
30 been suggested^{139,149,150}, they might also be candidates for disease biomarkers owing to their high levels of stability^{151,152}. Genes that undergo recursive splicing

have been linked to neurodevelopmental disorders¹⁴, but it remains to be seen whether variations in RS sites are involved in these diseases.

Non-canonical splicing in cancer. A prevalent feature of most cancer types is widespread intron retention^{131,153-155}. This could relate to competition for the spliceosome due to the high transcriptional activity in tumours, as limiting spliceosomal activity is a known cause of intron retention¹⁵⁶ (**Figure 6C**). Intron retention more often occurs in genes encoding RNA splicing and export factors, and therefore it may perturb the autoregulatory mechanisms of these genes in cancers. Moreover, enrichment in intron retention is associated with the presence of somatic single nucleotide variants in cancer, particularly in tumour-suppressor genes¹⁵⁴. Cancer-associated mutations in splicing factors such as U2 Small Nuclear RNA Auxiliary Factor 1 (U2AF1) or Splicing Factor 3b, Subunit 1 (SF3B1) can also promote intron retention or use of alternative 3' splice sites^{157,158}. It was proposed that mutations in SF3B1 induce selection of cryptic 3' splice sites through use of a different branch point^{95,157}, which leads to partial inclusion of the 3' end of the intron. Importantly, half of these aberrantly spliced transcripts are NMD-sensitive and lead to downregulation of corresponding mRNAs and proteins¹⁵⁷. Finally, differential splicing of several exons has been observed in breast cancer^{26,159}.

Genomic deletion breakpoints and chromosomal rearrangements that generate chimeric transcripts are another common feature of cancer. Notably, the same chimeric transcripts can also be detected at a lower level in non-cancer cells that do not contain the chromosomal rearrangement. In this case, trans-splicing generates the chimeric transcript. For example, *JAZF1-SUZ12* and *PAX3-FOXO1* chimeric transcripts are normally generated by trans-splicing of independent transcripts in both endometrial and mesenchymal stem cells, whereas chromosomal rearrangements result in fusions of these genes in endometrial stromal tumours and rhabdomyosarcomas, respectively^{86,132} (**Figure 4D**). This could reflect that parental genes have properties, such as spatial gene proximity or sequence features, that facilitate the trans-splicing of individual transcripts in wild-type cells and homologous recombination to cause chromosomal rearrangements in cancer. Alternatively, constitutive generation of trans-spliced

molecules in wild type cells might in some way facilitate the long-term chromosomal rearrangements observed in cancer by unknown mechanisms. Furthermore, cis-splicing between adjacent genes also commonly produces chimeric transcripts in cancer, such as the *SLC45A3-ELK4* transcript in prostate cancer^{160,161}. Taken together, these observations suggest that non-canonical splicing events such as intron retention and chimeric transcripts could have important roles in cancer.

Therapeutic opportunities. Splicing can be exploited for three types of therapeutic strategies: those that modify activity of splicing factors, those that change specific splicing events, and those that exploit non-canonical splicing mechanisms. The first holds particularly great potential in certain types of cancer, where genetic knockdown or pharmacological inhibition of spliceosomal components can prevent the growth and metastasis of MYC-driven tumours^{156,162,163}. In spite of these components being required in all cells, the increased demand for spliceosomal components induces accumulation of retained introns and increases apoptosis specifically in tumours (**Figure 6C**).

In cases in which specific splicing events need to be corrected, the pioneer studies restored normal splicing of β -globin in β -thalassaemia through the use of chemically modified antisense oligonucleotides (ASOs) that sterically block binding of the splicing machinery while avoiding RNase H-mediated degradation of the target RNA¹⁶⁴. This approach was successful in correcting splicing in SMA and many other diseases^{165,166}. Antisense sequences can also be delivered as modified U-snRNA molecules, using viral vectors that efficiently transfer a modified U-snRNA gene into the affected tissue, which allows continuous expression without the need of repetitive administration^{167,168}. Both ASOs and modified U-snRNAs can be directed either to splice sites, to branch points or to other regulatory elements, such as splicing enhancers or silencers, and therefore they were successful in preventing splicing of cryptic exons in a variety of diseases that are caused by deep-intronic mutations^{155,166-170}. To increase their efficiency, bifunctional ASOs or U-snRNAs can be designed, which contain an RNA binding domain and an effector domain, which recruits splicing factors that either enhance or silence splicing^{171,172}. Finally, therapeutic strategies based on

CRISPR-Cas9 genome editing have recently been successful to induce exon-skipping in vivo in adult mice¹⁷³⁻¹⁷⁵, indicating that this tool is likely to prove valuable as therapy to correct various types of canonical and cryptic splicing events in human diseases.

5 Several non-canonical splicing mechanisms can also be exploited as therapies. Trans-splicing has been applied to correct genetic mutations in monogenic disorders. In this technique, known as Spliceosome-Mediated RNA Trans-splicing (SMaRT), specific regions within the mutated mRNA are replaced using engineered RNA trans-splicing molecules as templates^{176,177}. These template
10 molecules contain the wild-type mRNA sequence to be replaced, a domain with the essential splicing elements and a domain that binds the target region. This strategy has been applied to many diseases, such as muscular dystrophies, haemophilia and cancer^{177,178}. Other types of non-canonical events might also prove useful for therapies, such as for example the designed artificial circRNAs
15 that could serve as aptamers, trans-cleaving ribozymes, small interfering RNAs (siRNAs), or as sponges to sequester micro RNAs (miRNAs) or RBPs^{151,179}.

Future perspectives

In this Review we have seen how new methods have led to the discovery of
20 various types of splicing events. The next challenge will be to systematically examine non-canonical splicing events that occur as a result of genetic variation, as this would clarify their importance from the perspective of evolution and disease. So far, many mutations affecting splicing have been identified by exome sequencing, which can only identify intronic mutations within a limited region
25 around the annotated exon-intron boundaries. Therefore, genome-wide sequencing will be required to reveal the full range of intronic variation that can activate cryptic splicing, perturb distal branch points or disturb regulatory regions³². It is also important to bear in mind that abnormally processed pre-mRNAs can interfere with transcription or cause co-transcriptional decay^{36,180}.
30 Dedicated genomic and transcriptomic experiments and computational approaches will therefore be needed to detect the full range of mutations that

cause disease via non-canonical splicing.

Even though it is clear that many non-canonical splicing events take place in human transcripts, our understanding of their roles in disease and physiology remains limited. We first need to better understand their roles in generating
5 alternative transcripts with modified stability, translation or localisation, production of new protein isoforms, or sequestration of specific RBPs and miRNAs. We also need to uncover their roles in diversifying tissue-specific or cell-specific patterns of gene expression across populations^{181,182}. Many non-canonical splicing events are enriched in the central nervous system, including
10 cryptic exons¹⁰, microexons¹², RS sites^{14,15} and circRNAs¹⁸³. Much remains to be learnt about how these mechanisms contribute to the complexity of gene regulation and the diversity of protein isoforms produced in the brain.

As the next round of ENCODE data on protein-RNA interactions becomes available, understanding of non-canonical splicing events that are hidden deep
15 within introns will be crucial to help explain those interactions for which a function has not yet been identified¹⁸⁴. Chromatin structure, DNA methylation, histone marks, nucleosome positioning and the kinetics of transcriptional elongation all contribute to splicing regulation in coordination with RBPs and the spliceosome¹⁸⁵. It remains to be seen if these factors cooperate in the control of
20 non-canonical splicing. It is likely that diverse regulatory interactions within intronic regions contribute to the quality control that prevents aberrant cryptic splicing from causing disease²⁷. Nevertheless, it is clear that many non-canonical splicing events escape this quality control, and their role as a source for new molecular functions during evolution will remain a fascinating subject of
25 research for many years.

Glossary

β -thalasaemia: A genetic blood disorder characterized by a defective synthesis of the β -globin chains of hemoglobin, thus causing abnormal erythropoiesis and anemia.

- 5 ***Alu* element:** A retrotransposon belonging to the family of short interspersed elements (SINE), consisting of an ~300 nt sequence, which originally derived from the 7SL RNA.

Aptamers: Oligonucleotide (or peptide) molecules that have secondary and tertiary structures that strongly bind to specific proteins or other cellular targets.
10

Ataxia telangiectasia: Autosomal recessive disorder involving cerebellar degeneration, immunodeficiency, chromosomal instability, radiosensitivity and cancer predisposition. It is caused by mutations in ATM gene.

- Autophagy:** Intracellular pathway responsible for regulated disassembly of unnecessary or dysfunctional cellular components after their targeting to lysosomes.
15

Axonogenesis: Generation and outgrowth of axons during neuronal development.

- CLIP:** A method used to identify the RNA targets bound by an RNA-binding protein-of-interest that employs crosslinking, immunoprecipitation and stringent purification of protein-RNA complexes by SDS-PAGE.
20

Chimeric transcript: Transcript formed when sections of two or more different genes are joined together in a new transcript either via splicing or as a result of chromosomal fusions.

- 25 **CircRNA:** RNA that has become circularised owing to intramolecular ligation of its 5' and 3' ends.

Co-transcriptional decay: RNA surveillance mechanism that acts in the nucleus while transcripts are still associated with the chromatin template.

Cryptic exon: An exon that is not annotated by the current genomic databases, such as ENSEMBL, and are often only revealed after removing a repressive RBP or after a genomic mutation that increases its splicing efficiency.

Duchenne muscular dystrophy: A progressive proximal muscular dystrophy caused by mutations in the dystrophin (*DMD*) gene.

Exitron: An intron located within an annotated exon.

Exon definition: The process by which exons are recognised and defined as functional units via interactions between multiple snRNPs and RBPs, especially U1 and U2 snRNPs and SR proteins.

Hyperphenylalaninaemia: A neurologic disorder caused by autosomal recessive mutations in the genes encoding enzymes involved in the synthesis or regeneration of BH4 cofactor. The most common form is caused by mutations in the *PTS* gene.

Intrasplicing: An unconventional splicing mechanism in which splicing to a 3' splice site reconstitutes a new 3' splice to be used in a subsequent splicing step.

Laron syndrome: Autosomal recessive disorder characterized by short stature that results from mutations in growth hormone (GH) receptor gene.

Microexon: Exon that is shorter than 30 nts.

NMD: Nonsense mediated decay, a pathway that initiates decay of certain transcripts, especially those containing a PTC.

NMD-exon: Exon that contains a PTC, and is therefore targeted for NMD.

NOVA: A joint name for RBPs encoded by two partially redundant genes that are expressed in the brain; neuro-oncological ventral antigen 1 and 2 (*NOVA1* and *NOVA2*).

PTC: premature termination codon

RBP: RNA-binding protein

RS: recursive splicing, a mechanism that which allows an intron to be spliced in two or more steps.

- 5 **RS exon:** An exon that follows an RS site and which is required for the exon definition mechanism that initiates splicing at the RS site.

RS site: The site of recursive splicing, which consists of a 3' splice site that is followed by a sequence that reconstitutes a 5' splice site after the first splicing event.

- 10 **Seed sequence:** The section of a sequencing read that is used to align the read to the genome or transcriptome.

snRNPs: Ribonucleoprotein complexes assembled around the small nuclear RNAs (snRNAs) that interact with splice sites or the branch point on pre-mRNA and thereby coordinate and catalyse the splicing reaction.

- 15 **Splice sites:** Sequences at the boundary of exons and introns, which contain motifs that recruit snRNPs and RBPs to initiate the splicing reaction. 3' and 5' splice sites are located upstream and downstream of exons, respectively.

Spliceosome: A macromolecular machine consisting of snRNPs and additional RBPs that coordinate and catalyse the splicing reaction.

- 20 **SR proteins:** A family of RBPs containing a protein domain with long repeats of serine and arginine that generally promote exon definition when binding to exons.

U2AF complex: Complex of two U2 auxiliary factor RBPs that bind the 3' splice site and facilitate the recruitment of the U2 snRNP to the branch point.

25

Figure legends

Figure 1: Cryptic exons and microexons. a) Many introns contain proximally spaced sequences that resemble splice sites, which can in some cases lead to splicing of 'cryptic' exons. Cryptic exons often introduce premature termination codons (PTCs), which may target the resulting transcripts for nonsense-mediated decay (NMD). Such NMD-exons are common within transcripts that encode splicing activators, where they function as part of autoregulatory mechanisms³³⁻³⁵. In this example, the SR protein enhances inclusion of an NMD-exon within its own mRNA as part of a negative autoregulatory feedback that maintains appropriate steady-state abundance. **b)** An *Alu* element is normally composed of two arms, which contain an A-linker and polyA tail. The *Alu* can become retrotransposed into the antisense strand relative to the gene, so that transcription of the gene produces antisense *Alu* sequence that contains two U-tracts at the beginning of each arm. Many such antisense *Alu* elements are capable of forming cryptic exons owing to the presence of splice site-like motifs³⁷. However, they are normally repressed by a hnRNP C tetramer (green circle), possibly because each U-tract can bind the two RNA Recognition Motif domains that are present on the opposite surfaces of the tetramer (as indicated by the green arrow)^{8,186}. The example provided here shows the U-tracts around the *Alu* exon from the *CD55* gene (encoding CD55 molecule). Below, mutations in the U-tracts are shown that decrease binding of hnRNP C, allowing binding of U2 small nuclear RNA auxiliary factor (U2AF2) and TIA1 cytotoxic granule-associated RNA binding protein (TIA1), which initiate splicing of a cryptic *Alu* exon^{8,37,39}. C = hnRNP C protein. **c)** Microexons can be detected from gapped regions in sequencing reads^{11,13,44}. After mapping of multiple parts of the sequence read to flanking exons, unmapped intervening sequences are aligned to the intronic sequence present between the two exons, with preference given to those that are flanked by conserved splice site motifs. Inclusion of microexons can be enhanced by RNA binding proteins (RBPs) such as Serine/Arginine Repetitive Matrix 4 (SRRM4), an SR protein that binds upstream of microexons and promotes microexon splicing. Inclusion of microexons typically leads to modulation of overlapping or adjacent protein domains to change protein

activity. SRRM4 is reduced in autism patients leading to decreased inclusion of microexons¹². YAG, 3' splice site; GU, 5' splice site; NMD, Nonsense-mediated decay; μ ?, possible microexon; μ , microexon.

Figure 2: Recursive splicing of long introns. a) Total RNA-seq read counts

display a characteristic pattern of depletion from the start to the ends of long introns, which can be used to infer exon positions and splicing events^{47,48}. “Saw-tooth” patterns that overlap novel junction reads indicate splicing at deep intronic loci and are candidates for recursive splicing^{14,15}. Here, the upstream exon first uses a 3' splice site to remove the first part of the intron. This process reconstitutes a 5' splice site that can then be used to remove the next section of the intron. This special type of splice site that is shown in the weblogo is referred to as a recursive splicing site (RS site). **b)** Recursive splicing in vertebrates requires the RS site to overlap a cryptic ‘RS exon’, which initiates the exon definition mechanism, required for recognition of the 3' splice site of the RS site¹⁴. After the first splicing step, the 5' splice site of the RS site competes with the 5' splice site of the RS exon. In the second step of splicing, the outcome of this competition decides whether the RS exon is skipped owing to recursive splicing, or included as an NMD-exon. While the preceding exons from major isoforms end in sequences that favour RS exon skipping, the minor isoforms and cryptic elements end in sequences that favour RS exon inclusion. RS site, Recursive splice site; RS exon, Recursive splicing exon; YAG, 3' splice site; GURAG, 5' splice site.

Figure 3: Intron retention and exitrons. a) Intron retention events are

detected as an accumulation of reads across intronic regions, or increases in the ratio of exon-intron reads to exon-exon reads²¹⁻²⁵. Intron retention events are characterised by numerous features including weak splice sites, high GC content and short intron lengths. Trans-acting factors such as RBPs, the spliceosome and the EJC can also regulate specific intron retention events. The resulting transcripts are typically either retained in the nucleus or targeted for NMD in the cytoplasm or may result in truncated proteins^{21,53,55}. Other intron retention

events might be translated into truncated proteins. **b)** Exitrons are introns within annotated protein-coding exons that can be removed owing to the presence of internal splice site motifs within the exon^{25,26}. Exitron-containing exons are longer than typical exons, and removal of the exitron can lead to changes in protein structure or degradation via NMD. NMD, Nonsense-mediated decay; AG, 3' splice site; GU, 5' splice site.

Figure 4: Formation of circRNAs and chimeric transcripts. **a)** CircRNAs are produced by head to tail splicing and can be both mono- or multi-exonic. In this multi-exonic example the 3' splice site of an upstream exon becomes spliced to the 5' splice site of a downstream exon to generate a circular transcript that either has the intervening intron removed (exonic circRNA) or retained between the two circularized exons (intron-exon circRNA)²⁰. Their formation is promoted when the pre-mRNA regions flanking the exon termini are brought in proximity. This can be due to the action of RNA-binding proteins such as Quaking (QKI) or muscleblind-like (MBNL), which bind to flanking regions^{74,75}. Alternatively, this can be due to RNA hybridisation of the flanking regions, which can be caused by *Alu* elements in primates⁷⁰. **b)** Circular RNAs are resistant to RNase R, which can be used for their enrichment during preparation of cDNA libraries. They can then be detected in sequencing data by junction reads that are in a head-to-tail orientation¹⁶⁻¹⁹. **c)** Chimeric RNA products can also be produced by cis-splicing when transcript termination is deficient⁷⁶. This process results in read-through of one gene into its neighbouring gene, before splicing occurs between the penultimate exon of gene 1 and the second exon of gene 2, which is seen in the *CTSC-RAB38* genes in cancer. **d)** Trans-splicing occurs when exons of two different transcripts become spliced together⁸⁰⁻⁸⁷. Alternatively, the same chimeric transcripts can be produced when genes become fused at the level of the DNA, such as in *JAZF1-SUZ12* genes in some cancer, which leads to the same chimeric transcript being produced by a linear splicing reaction. RBP, RNA-binding protein.

Figure 5: A summary of human splice site consensus motifs. Summarised splice site sequences are classified using the nucleotides marked by the grey boxes. All borders of human exons within Ensembl v83 multi-exon transcripts that overlap with RefSeq mRNA IDs were used. Identical coordinates from overlapping transcripts were collapsed into a single occurrence such that junctions were not counted multiple times. First exons had only their exon-intron junction evaluated, whilst terminal exons had only their intron-exon junction evaluated. This led to a total of 189,255 5' splice sites (shown on the left, with the line showing exon-intron border) and 187,091 of 3' splice sites (shown on the right, with the line showing intron-exon border). U12-type splice site sequences were obtained from U12DB¹⁸⁷. After identifying the 5' and 3' sites overlapping with the U12-type splice sites, respectively, the remaining U2-type splice site sequences were examined. 5' and 3' splice sites were classified independently and sequentially based on the indicated nucleotides. For example, 53.58% of unique U1-type exon-intron junctions contain GTRAG, and the remaining U1-type junctions were classified based on the first two intronic nucleotides. The percentage of unique junctions containing each motif are indicated. Weblogo 3 was used to show the relative frequency of nucleotides at each position¹⁸⁸. **a)** The U1-type 5' splice sites with GT at the border, and U2-type 3' splice sites with AG at the border, **b)** The U11-type 5' splice sites and U12-type 3' splice sites, **c)** The U1-type 5' splice sites with GC at the border, remaining U1-type 5' splice sites with TN at the border, where N stands for any nucleotide, U1-type 5' splice sites with VN at the border, where V stands for any nucleotide except T, U2-type 3' splice sites with BG at the border, where B stands for any non-A nucleotide, the U2-type 3' splice sites with W at the border, where W stands for T or A.

Figure 6: Cryptic splicing in disease and therapeutic strategies. **a)** Cryptic exons are normally repressed by RBPs such as hnRNPC (green circle) or by U1 snRNP. **b)** Examples of mutations (numbered) in deep intronic regions that can activate cryptic splicing events in disease-associated genes. (1) hnRNPC (green circle) binding to a U-tract upstream of an antisense *Alu* element represses recognition of the cryptic 3' splice site within the element. Intronic deletions or

point mutations that shorten U-tract can impede hnRNP C recruitment but allow U2AF2 (shown in purple) binding, leading to *Alu* exonisation. A deletion within an *Alu* in the *PTS* gene (encoding 6-Pyruvoyltetrahydropterin Synthase) leads to splicing of an *Alu* exon that introduces a frameshift, thereby causing the neurologic disease hyperphenylalaninaemia^{8,141}. (2) In the *ATM* gene, U1snRNP (orange circle) binding to an intronic element within a cryptic exon inhibits its recognition as a splicing competent exon. Patients with ataxia telangiectasia present a 4 nt deletion that abolishes U1snRNP interaction, causing cryptic exon activation¹¹⁰. (3) A point mutation within a deep intronic sequence of the *CFTR* gene generates an active 5' splice site that allows insertion of a cryptic exon within the *CFTR* transcripts, which causes cystic fibrosis¹³⁵. (4) In the *BRCA2* gene, a point mutation that disrupts a canonical 3' splice site activates (depicted by a grey arrow) an upstream cryptic exon¹³⁶. Disrupted *BRCA2* expression causes breast, ovarian and other cancer types. **c)** New therapeutic strategies in cancer involve spliceosome targeting^{156,162,163}. In MYC-driven tumours, oncogenic MYC causes transcriptional amplification, which overloads the splicing machinery and makes these cells more sensitive to alterations in splicing fidelity. Genetic knockdown or pharmacological inhibition of spliceosomal components leads to accumulation of retained introns that results in increased apoptosis and reduced tumorigenic and metastatic potential of MYC-driven tumours. C, hnRNP C protein; U1, U1 snRNP; AF2, U2AF2 protein.

Box 1: Identification of non-canonical splicing events

High-throughput methodologies, and in particular RNA-seq, have created opportunities for transcriptome-wide annotation of rare, cell-type-specific transcripts and non-canonical splicing events in our transcriptomes^{12,14,15}. Whilst cDNAs generated from poly(A)-purified RNAs in mRNA-seq primarily detect fully spliced mRNAs that have passed the cellular quality control, cDNAs generated with random-primers in total RNA-seq also identified the intermediate steps of splicing reactions^{14,15,48,189}. To study non-canonical splicing, several alignment algorithms have been tailored for the discovery of novel splice junctions with

RNA-seq data^{11,29,30}. A particularly great diversity of transcripts was found in the brain, in agreement with the great variety of cell types and functions that exist in this organ^{10,14,29,30}. Even though many of these are rare and non-functional, some are functionally important non-canonical splicing events. One way to distinguish those that may have a function is to focus on novel junctions that contact conserved sequences that bear features of splicing elements, such as proximally spaced 3' and 5' splice sites (i.e. within typical exon size limits), branch points, exonic enhancers and other regulatory elements^{10,14,26,31}.

More specialised methods for preparing and analysing RNA-seq data have led to discovery of new types of exons and RNAs. For example, several commonly used alignment algorithms require a minimum length of the seed sequence for the alignment, which generally limits detection of exons to those longer than 30 nt, thereby excluding microexons. To overcome this limitation, alignment algorithms were modified to use shorter seeds and to allow longer reads to be mapped in multiple parts¹¹. If much shorter parts of a long read are mapped to two exons in a way that leaves an unmapped intervening sequence, this sequence can then be mapped back to the intronic sequence present between the two exons, with priority given to conserved sites flanked by proximally spaced 3' and 5' splice sites consistent with a <30nt microexon **(Figure 1B)**^{11,13,44}. Alternatively, custom alignment files incorporating all putative cryptic exons with flanking splice sites can be used for mapping¹².

Information on splicing efficiency can also be gained by analysis of intronic reads. For example, intron retention can be examined by the ratio of exon-intron junction reads relative to junction spanning reads, or by comparing read coverage across the intron to the flanking exons^{21,23,24}. Moreover, co-transcriptional splicing patterns can be visualized across introns in total RNA-seq data as 'saw-tooth' patterns^{14,15,31,48}. Specifically, the RNA abundance at the start of a long intron is higher than at its end owing to the presence of nascent transcripts in various stages of transcription, and because splicing can't proceed until transcription of the 3' splice site^{47,48}. Novel junctions that overlap clear troughs in the co-transcriptional splicing patterns often identify recursive splice sites (RS sites)^{14,15} **(Figure 2A)**.

Dedicated computational approaches also facilitated discovery of circular RNAs (circRNAs) and chimeric transcripts^{66,67,190,191}. In the simplest method for discovery of circRNAs, unaligned reads are split into two parts before being re-mapped to exons. If the second part maps to an exon upstream of the first part, these are then considered as circRNA candidates¹⁷ (**Figure 4B**). This local re-ordering of the alignments distinguishes circRNAs from chimeric transcripts that can also be identified by discordant alignments^{192,193} (**Figure 4B-D**). Experimentally enriching the sample preparation for non-linear RNAs before cDNA library preparation using the exoribonuclease RNase R can further enhance circRNA discovery^{65,67}.

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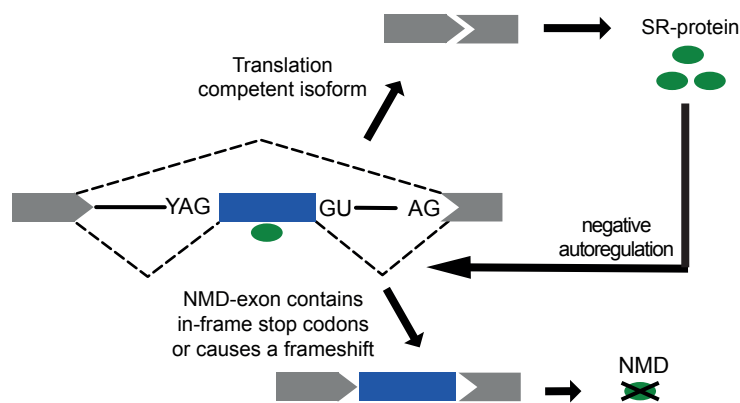
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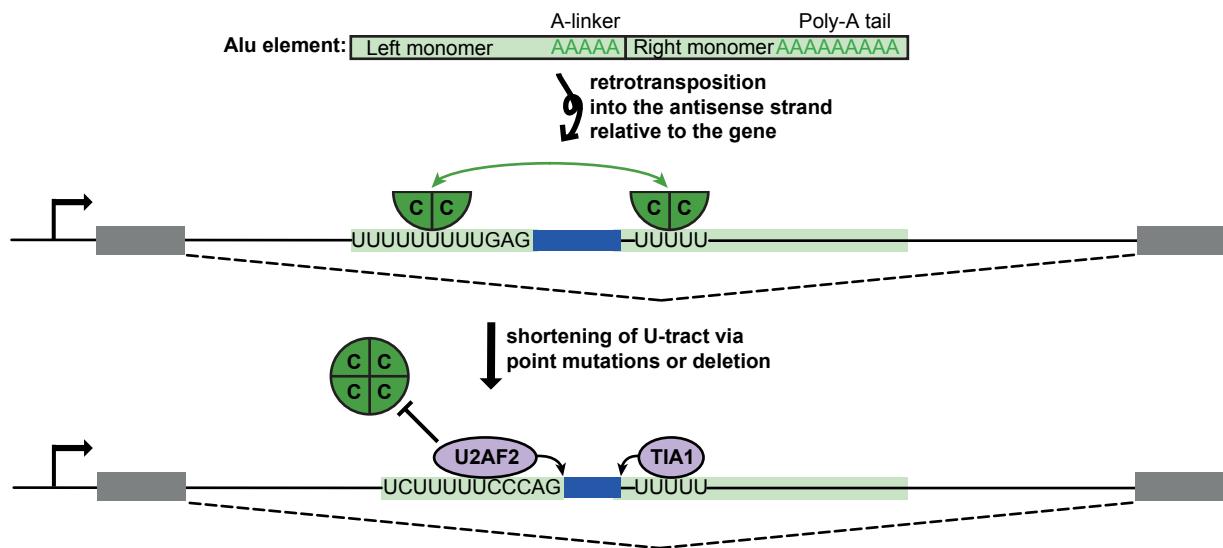
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Figure 1

A.



B.



C.

How to find the correct alignment to a microexon?

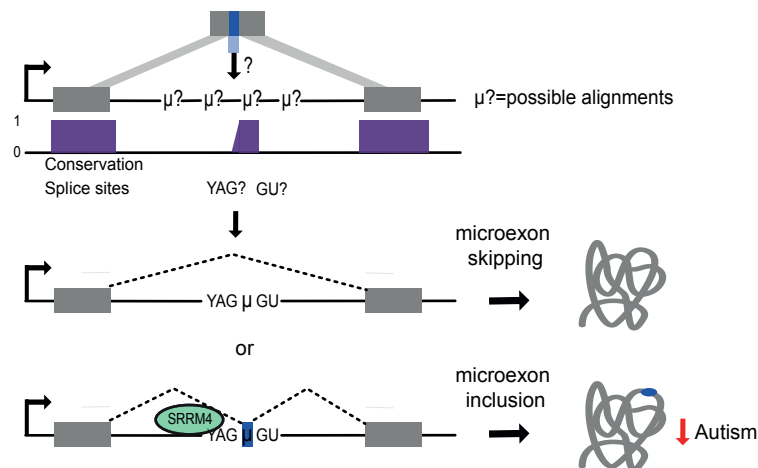


Figure 2

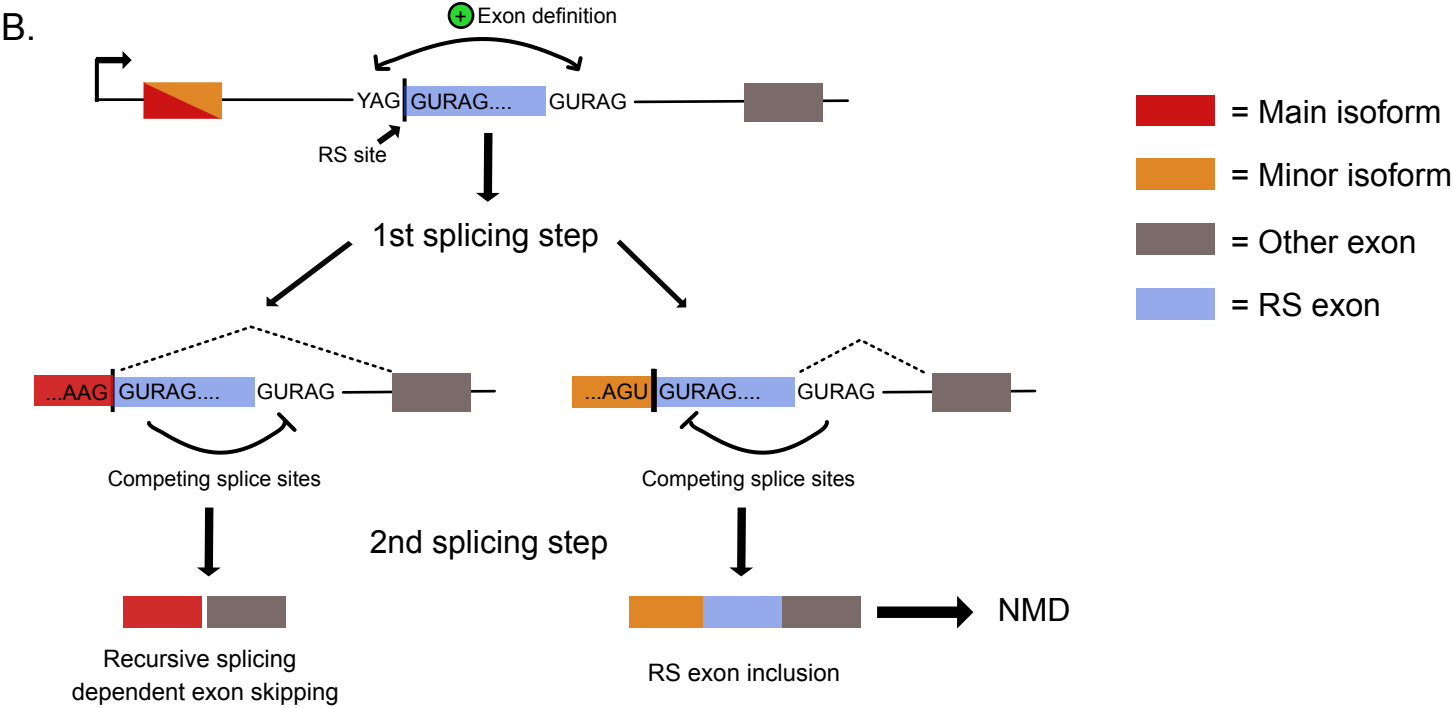
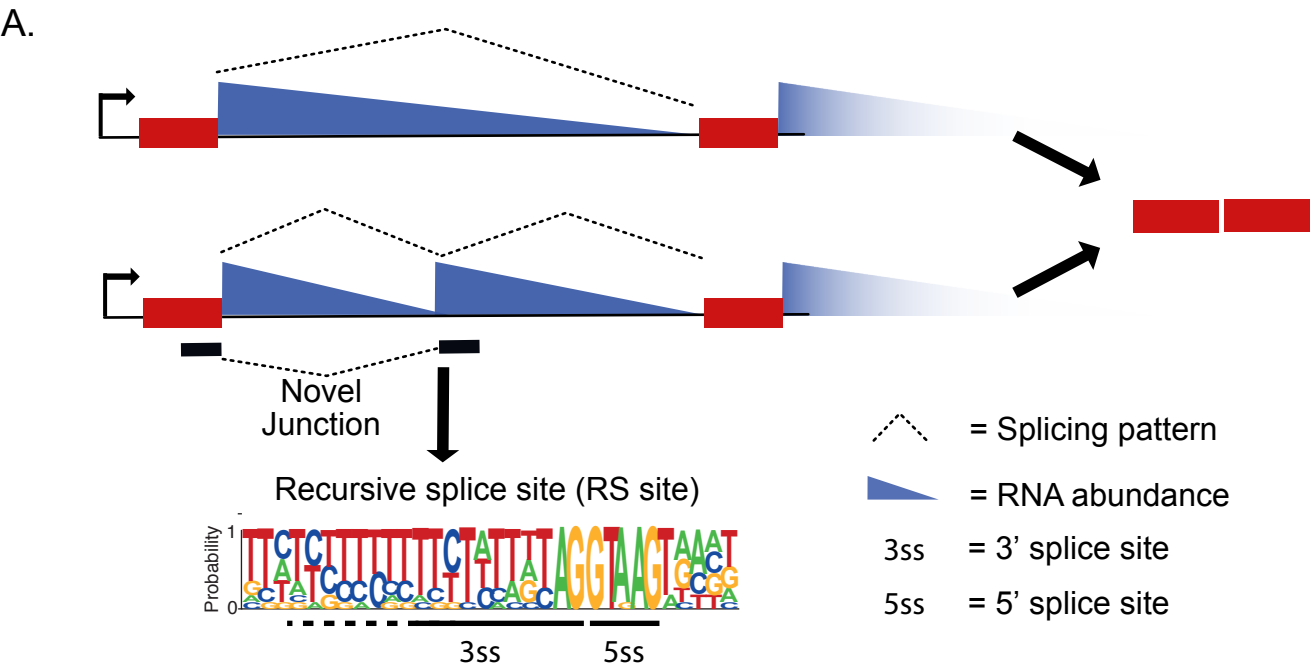
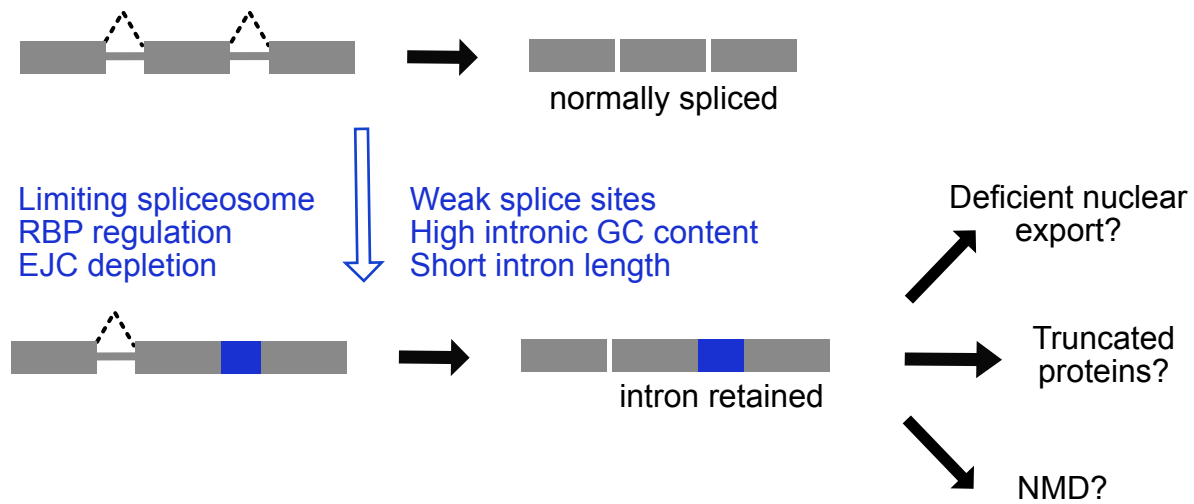


Figure 3

A.



B.

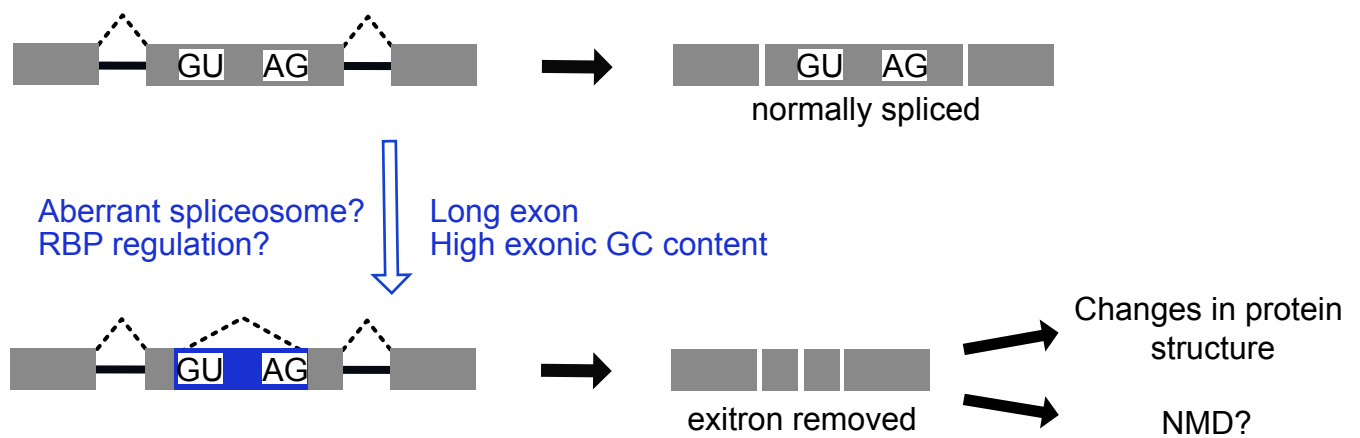


Figure 4

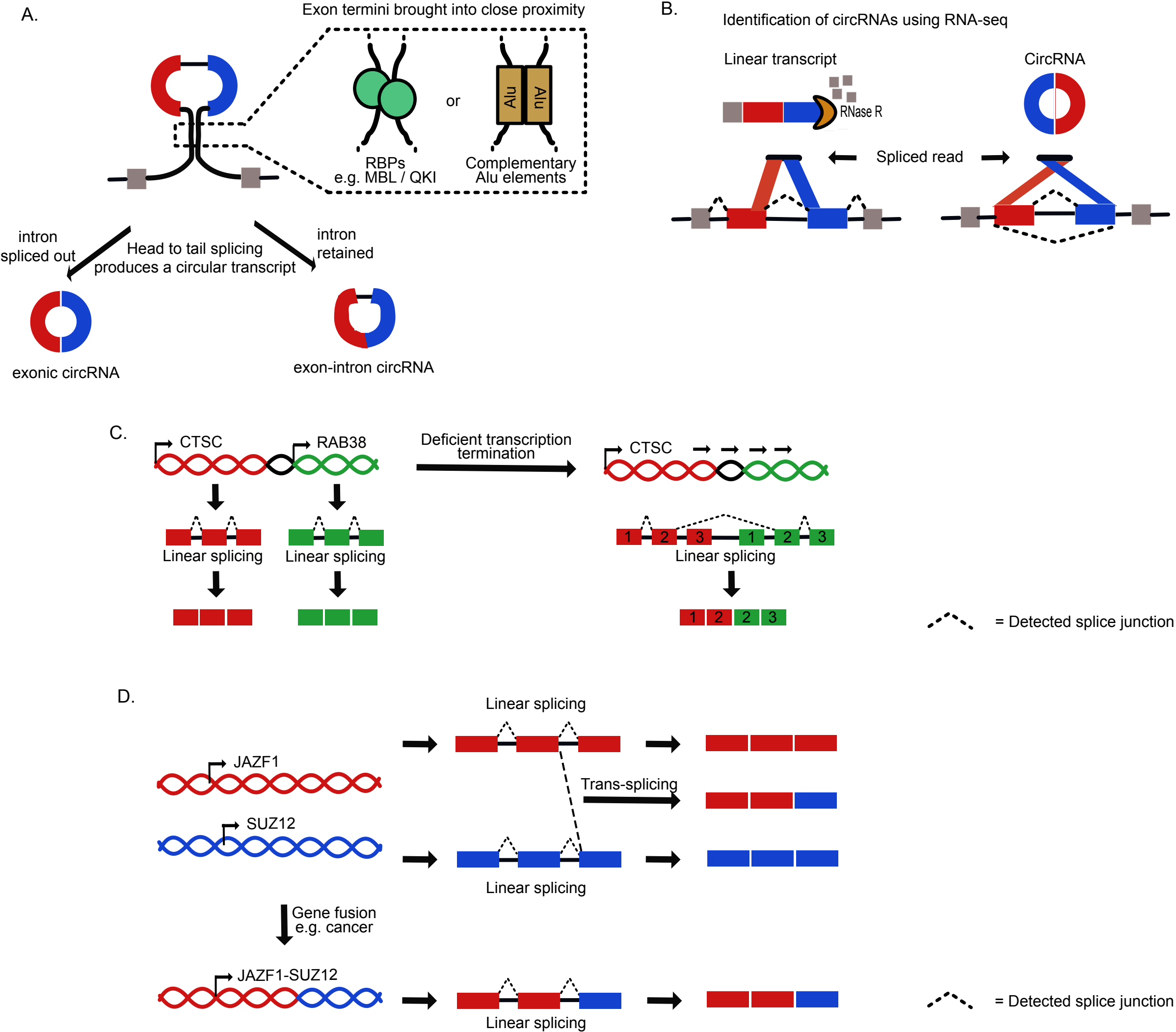
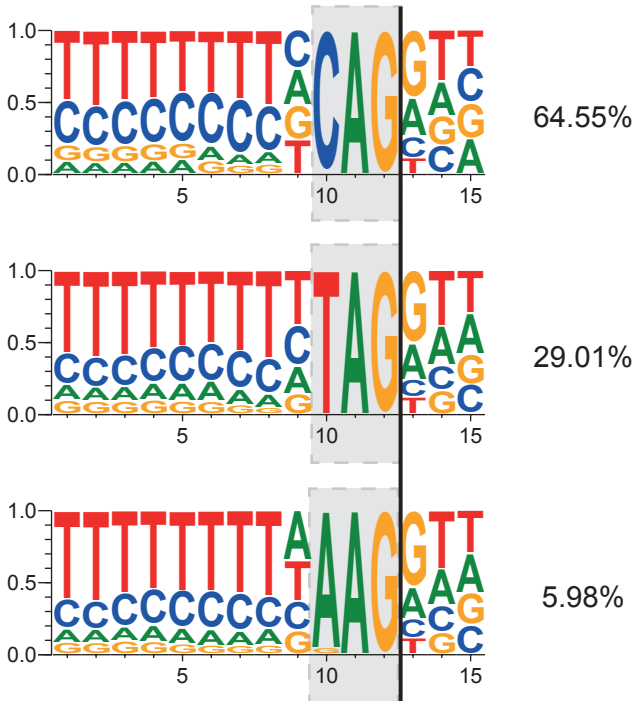
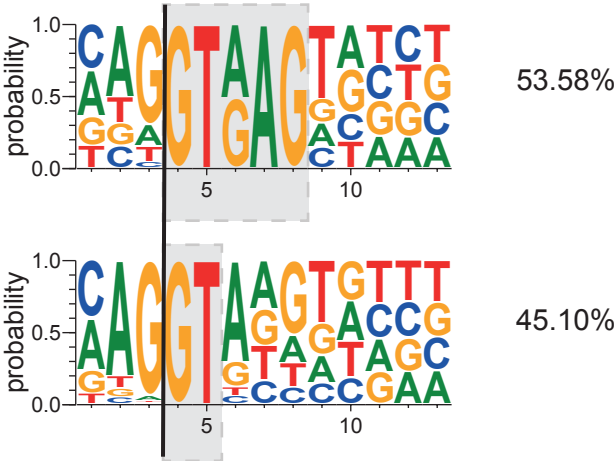


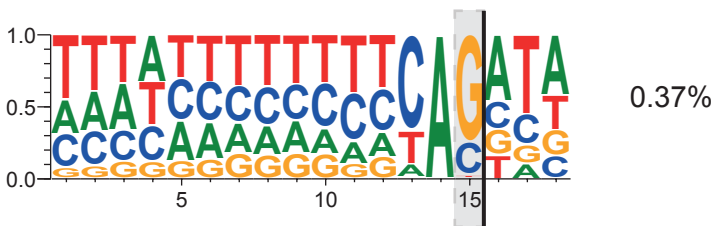
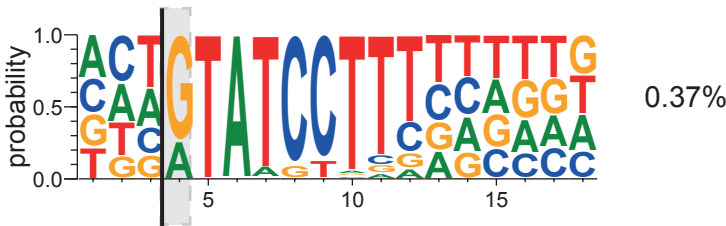
Figure 5



A. GT-AG splice sites



B. U12-type splice sites



C. Atypical splice sites

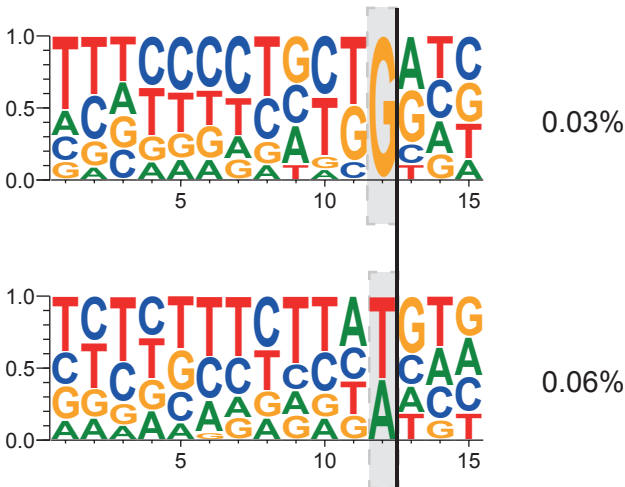
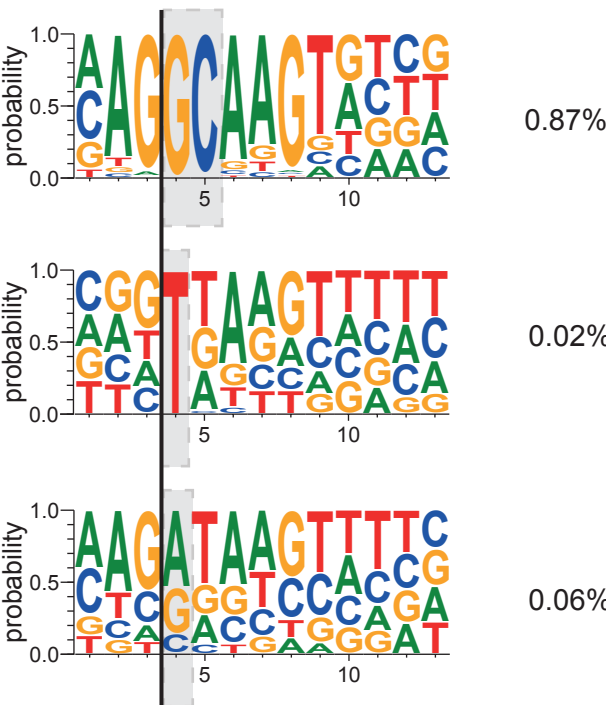
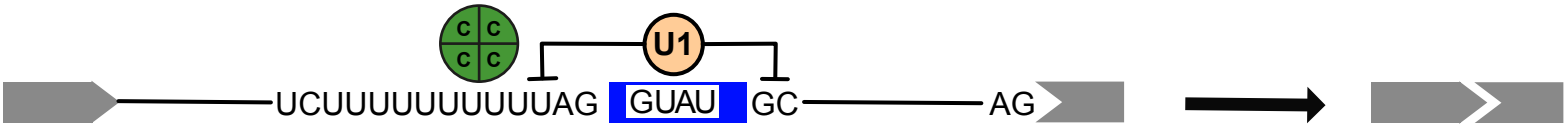


Figure 6

A. Repressive mechanisms prevent splicing of cryptic exons



B. Four types of disease-causing mutations that can activate splicing of cryptic exons

